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PROBES FOR CHONDROGENESIS

[0001] This invention claims priority to U.S. provisional application number 60/211,384, filed June 14, 2000.

[0002] This invention is supported, at least in part, by Grant Nos. RO1AR046196 and P60AR020618 from the National Institutes of Health. The Federal Government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] This invention relates to probes for detecting chondrogenesis.

BACKGROUND

[0004] Cartilage is a dense connective tissue that comprises part of the skeleton in adult humans. Cartilage provides support and attachment points for body structures, protects underlying tissues, and provides structural models in which many bones develop.

[0005] Cartilage is largely comprised of cells, called chondrocytes, embedded in an extracellular matrix. The extracellular matrix mainly consists of collagen type II and proteoglycans, the components of which are exuded into the intercellular space by the chondrocytes, where they are assembled to form macromolecules. The chondrocytes make up about 5% of the volume of the cartilage tissue of an adult individual.

[0006] Chondrocytes are formed by differentiation of mesenchymal progenitor cells (MPCs), also called mesenchymal stem cells (MSCs). These cells form chondrocytes during embryonic development (see below). In addition, MPCs are found in many tissues of the adult body, and are multipotent in that they can differentiate into a number of different cell types (see

Fig. 1). The process of differentiation from progenitor or stem cell to chondrocyte is called chondrogenesis.

Chondrogenesis in Development and Growth

[0007] Chondrocytes are an essential component to bone development and growth, a process called endochondral ossification. For example, most bones of the human skeleton develop from masses of hyaline cartilage. This cartilage is formed by chondrocytes that are differentiated from MPCs. The first part of chondrogenesis is the formation of a precartilage condensation, where the MPCs come together through cell-cell interactions and there is some proliferation. After condensation, the progression phase of chondrogenesis occurs, with cells beginning to produce molecules characteristic of cartilage, such as type II collagen. There is expansion of the cartilaginous tissue by production of large amounts of extracellular matrix containing mainly collagen and proteoglycans. This leads to the formation of a cartilage anlagen for a given bone.

[0008] In certain regions of this cartilage, the chondrocytes further differentiate into hypertrophic chondrocytes that secrete bone-related molecules and their surrounding extracellular matrix is calcified. The cells then die by apoptosis (programmed cell death). Blood vessels invade the calcified cartilage and osteoblasts (the cells responsible for bone formation) are attracted. Chondroclastic cells remove the cartilage and the osteoblasts lay down new bone. This bone is later remodeled by osteoclasts and osteoblasts to form mature bone.

[0009] Other regions of the cartilage anlagen are not removed during development; specifically the growth plates and the articular cartilages. Growth plate chondrocytes enter a programmed pathway in which they proliferate for some time and then become hypertrophic and die, with replacement of the hypertrophic region by the mechanism described above, involving blood vessel invasion, etc. This mechanism allows bones to grow longitudinally until puberty.

[0040] Articular cartilage resembles the growth plate in the neonatal stage, but eventually there is formation of a cartilage in which the chondrocytes no longer proliferate or hypertrophy – a permanent cartilage. Articular cartilage is responsible for weight-bearing and shock absorption in joints. It is the cartilage that breaks down in degenerative arthritic diseases. Other permanent cartilages include those that form rings in the trachea or the cartilage of the nose and ears.

[0011] Because it is important, in certain instances, to monitor progression of chondrogenesis in both formation of cartilage and bone, there is a need for markers and probes to detect and ascertain the extent of these processes.

Chondrogenesis in Natural Repair and Regeneration

[0012] In addition to formation of bone during development, chondrocytes are also involved in the formation of bone in repair of bone fractures. Within a typical fracture site, there is both intramembranous bone formation (where there is no cartilage intermediate) and endochondral bone formation (in which cartilage is first formed and then replaced by bone, in a manner similar to that seen in development).

[0013] Immediately after a fracture, a fibrous clot is formed and granulation tissue results as macrophages and other cells invade it. This is called the "external callus." Bone begins to be made by osteoblasts adjacent to the fracture, forming a hard callus. At the same time, progenitor cells from the surrounding tissues proliferate and begin to differentiate into chondrocytes within the granulation tissue. This cartilaginous callus is later replaced by bone tissue, similar to the process in which hyaline cartilage of a developing bone is replaced.

[0014] Because the endochondral component is important to effect proper bone repair, there is a need for markers and probes to detect and ascertain the extent of chondrogenesis in this process. This is especially true when a so-called non-union occurs, in which the fracture does not heal. Understanding what stage the repair has reached would aid in the choice of the remedial treatment.

Chondrogenesis in Therapeutic Cartilage Repair and Regeneration

[0015] In a separate application, the use of implanted mesenchymal progenitor cells to produce repair of cartilaginous tissues would benefit from knowledge of the stage of differentiation that the cells have reached. For example, in articular cartilage repair, the undifferentiated progenitor cells, either injected or implanted into area of the body where there is defective cartilage, is a possible treatment modality. The course of such treatment includes the sampling (biopsy) of repair tissue at some time after implantation. In order to aid in decisions

regarding the treatment, it is important to know the stage of chondrogenic differentiation that the implanted cells have reached. Thus, there is a need for markers and probes of chondrogenesis to detect and ascertain the extent of this process.

Chondrogenesis *In Vitro*

[0016] In addition to the involvement of chondrocytes in natural body process, manipulation of chondrocytic precursors *in vitro* is becoming increasingly important for "tissue engineering" methodologies.

[0017] For example, a population of MPCs can be manipulated *in vitro* such that a majority of cells become chondrocytes (see U.S. Pat. No. 5,908784 by Johnstone et al.). One use of such systems is to correct and repair cartilage defects through implantation into humans of such chondrocytes derived from differentiation of MPCs *in vitro* (for example, see U.S. Pat. No. 6,242,247 by Rieser, et al.).

[0018] Because tissue-engineered cartilage is a possible treatment for cartilaginous defects, there is a need for probes and detection methods to ensure that mesenchymal cells have differentiated into chondrocytes during the *in vitro* production of the cartilage.

Differentiation of MPCs as Related to Cancer

[0019] Chondrosarcoma is the second most common form of bone malignancy. These are generally slow growing sarcomas that are of unknown etiology and the cell type that initiates the formation of a chondrosarcoma within a bone is not known. Such cells, however, are characterized by the production of cartilage within the sarcoma by cells that differentiate into chondrocyte-like cells. In a specific type of chondrosarcoma, called "mesenchymal chondrosarcoma," cells of the tumor comprised all differentiation stages between and including MPCs and hypertrophic chondrocytes (Aigner, et al., 2000, Am J Pathol, 156:1327-35.).

[0020] Conventional chondrosarcoma tumors are graded from stage I through stage III, stage III being the most advanced. Such grading of chondrosarcomas is important for proper diagnosis and treatment of the condition. However, diagnosis and grading of chondrosarcoma has been problematic. For example, the criteria used to distinguish benign enchondroma from

low grade chondrosarcoma include parameters which are difficult to quantify such as increased cellularity and more than occasional binucleate cells. These histologic criteria are not absolute, and the diagnosis is frequently made by taking into account clinical features such as pain, rate of growth, location, and radiologic features.

[0021] Because it is difficult to stage these sarcomas, there is a need for probes and better detection methods to aid in the definition of the grade of a given chondrosarcoma to assist in the decision-making process for treatment.

SUMMARY OF THE INVENTION

[0022] The present invention provides new markers which can be used for detecting and staging chondrogenesis in cells. In one aspect, the markers are isolated polynucleotides, referred to hereinafter as CZF-1 and CZF-2, and fragments thereof. In one embodiment, the CZF-1 polynucleotide comprises the sequence set forth in SEQ ID NO. 1. In one embodiment, the CZF-2 polynucleotide comprises the sequence set forth in SEQ ID NO. 3. In another aspect the markers are antibodies which are immunospecific for the proteins encoded by CZF-1 or CZF-2.

[0023] The present invention also provides methods which employ the present markers to identify cells that have begun to differentiate into chondrocytes. Such cells are referred to hereinafter as "cells of interest". In one aspect, the method involves contacting the CZF-1 polynucleotide or a fragment thereof, or the CZF-2 polynucleotide or a fragment thereof, with RNA that has been extracted from or, alternatively, contained within the cells of interest, and assaying for the presence of a hybridization product between the polynucleotide and the RNA. In another aspect, the RNA is reverse transcribed and amplified using primers that have been derived from the CZF-1 or CZF-2 polynucleotides. In a further aspect, the method comprises contacting the cells of interest with antibodies that are immunospecific for the CZF-1 protein or the CZF-2 protein and assaying for the formation of an antigen-antibody complex between the anti-CZF-1 or anti-CZF-2 antibody and a protein in the cell.

[0024] The present invention also relates to the CZF-1 protein and the CZF-2 protein. Such proteins are useful for preparing antibodies that are used in the present methods for characterizing cells. The present invention also relates to polynucleotides or oligonucleotides

whose sequences are complementary to the coding sequences of CZF-1 or CZF-2, or regions thereof. Such polynucleotides and oligonucleotides are useful as probes or primers.

DESCRIPTION OF THE FIGURES

[0025] The present invention may be more readily understood by reference to the following figures wherein:

[0026] **Figure 1** is a diagram of MPC differentiation along different cell lineages; and

[0027] **Figure 2** is a diagram of differentiation of MPCs into chondrocytes; and

[0028] **Figure 3** is the DNA sequence of the full-length CZF-1 gene (SEQ ID NO. 1); and

[0029] **Figure 4** is the predicted amino acid sequence of the CZF-1 protein (SEQ ID NO. 2); and

[0030] **Figure 5** is the DNA sequence of the full-length CZF-2 gene (SEQ ID NO. 3); and

[0031] **Figure 6** is the predicted amino acid sequence of the CZF-2 protein (SEQ ID NO. 4); and

[0032] **Figure 7** is a diagram of the structure of the CZF-1 gene; and

[0033] **Figure 8** contains charts of expression of CZF-1, collagen type II and collagen type X in MPCs undergoing chondrogenesis; and

[0034] **Figure 9** is a Northern blot analysis of CZF-1, CZF-2 and Sox-9 expression in various tissues of humans; and

[0035] **Figure 10** is a Northern blot analysis of CZF-1 and CZF-2 in developing mouse embryos; and

[0036] **Figure 11** shows expression of CZF-1 in a 10.5 dpc mouse embryo.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0037] Unless otherwise indicated, the following terms used in this document have the following meanings:

[0038] "Transcription factor" refers to a protein that interacts, either directly or indirectly through other proteins, with RNA polymerase II to initiate transcription. Some transcription factors have the ability to bind to specific nucleotide nucleotide sequences within DNA.

[0039] "Zinc-finger" refers to one or more motifs within a protein that bind to Zn2+, such binding causing arrangement of the motif such that an α-helix within the motif can fit into and bind to the major groove of the DNA.

[0040] "Mesenchymal cells" refers to cells that can differentiate into a variety of differentiated cell types, including bone, bone marrow, cartilage, muscle, tendons and ligaments, and connective tissues.

[0041] "Chondrocytes" refers to cells that comprise cartilage.

[0042] "Chondrogenesis" refers to formation of cartilage, more specifically, to formation of chondrocytes from mesenchymal progenitor cells.

[0043] The present invention relates to isolated polynucleotides and oligonucleotides that are useful for characterizing the extent of chondrogenesis in cells of interest. In accordance with the present invention, the polynucleotides and oligonucleotides are derived from two genes, CZF-1 and CZF-2, that are transiently expressed during the early stages of chondrogenesis.

Discovery and Isolation of CZF Genes

[0044] A cell culture system that facilitates chondrogenic differentiation of postnatal mammalian marrow mesenchymal progenitor cells (MPCs), in a defined medium that includes

dexmathasone and TGFβ-1, was the source of RNA that was used for isolation of CZF-1 and -2 cDNAs (Johnstone, et al., 1998, Exp Cell Res, 238:265-72 and U.S. Pat. No. 5,908784).

[0045] In this *in vitro* differentiation system, MPCs progress through a number of stages which are defined by expression of various markers (see Fig. 2). Expression of collagen type II is a marker of chondrocytes and its expression begins to be seen after the cells of this system are in culture for 4 days. Collagen expression levels continue to increase thereafter. In this system, the cells continue to differentiate to form hypertrophic chondrocytes, which are marked by expression of collagen type X.. Collagen type X expression begins to occur on day 6, but doesn't reach high levels until after day 7.

[0046] RNA was prepared from the cells after 3 days in culture and is used to construct a cDNA library in the vector λgt10. This cDNA library was screened using a ^{32}P-labeled degenerate oligonucleotide probe containing all possible permutations coding for the sequence of amino acids, HTGEKP.

[0047] The HTGEKP sequence is a sequence motif common to proteins of the class called "zinc-finger" proteins. Such zinc-finger proteins have the ability to bind to DNA and these proteins have been shown to be transcription factors. Zinc-finger transcription factors of the Cys_2His_2 type are characterized by tandem arrays of sequence conforming approximately to the motif (Tyr, Phe)-X-Cys-X_{24}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His, where X represents any essential amino acid. The linker that connects adjacent zinc-finger domains is well conserved and has the consensus sequence His-Thr-Gly-Glu-Lys-Pro (HTGEKP). Therefore, a degenerate oligonucleotide probe representative of all possible codons encoding HTGEKP will hybridize to cDNA clones within the library encoding zinc-finger motifs.

[0048] Approximately 60,000 λ plaques were screened and plaques hybridizing to the degenerate HTGEKP probe were obtained. The cDNA inserts of these hybridizing clones were amplified using the polymerase chain reaction (PCR), cloned by the TA-tailing method, and the DNA sequence of the inserts was obtained. The obtained DNA sequences were compared to the NCBI nucleotide sequence database using the BLAST algorithm. The BLAST search showed that the one of the inserts (called CZF-1; Cartilage Zinc Finger) matched a 717 amino acid open reading frame (ORF) encoding a protein containing approximately 16 zinc-finger domains,

within a 3.6 Mb region in 19q13.4. Additional sequence encoding the amino acid terminal region of the protein is obtained by performing a 5' RACE procedure. The final protein sequence was found to be 717 amino acid residues in length.

[0049] The BLAST search showed that the other insert (called CZF-2) contained a 518 amino acid ORF containing approximately 12 zinc-finger domains.

Characterization of CZF Gene Expression

[0050] As stated earlier, in the *in vitro* system for differentiation of MPCs to chondrocytes, differentiation begins to occur after approximately 3 days in culture. This is shown in Fig. 8, which is RT-PCR amplification of CZF-1, collagen type II and collagen type X RNAs isolated differentiating MPCs on the days indicated. As indicated in Figure 8, collagen type II begins to be expressed at around day 5 and is a marker of chondrocytes. In this system, the cells further differentiate to "hypertrophic" chondrocytes, as indicated by expression of collagen type X, which is not present at significant levels until after 7 days in this system.

[0051] In this cell system, CZF-1 expression begins on day 4-5 in culture, is maximal on day 6, and begins to decrease thereafter. Therefore, CZF-1 begins to be expressed earlier than does collagen type II and is a marker for formation of chondrocytes.

[0052] Expression of both CZF-1 and -2 was examined in multiple tissues obtained from humans (see Fig. 9). Northern blot analysis of CZF-1 showed a restricted pattern of expression, with high mRNA levels in testis and thyroid. Northern blot analysis of CZF-2 showed high level expression in testis, thyroid, adrenal gland and placenta. Since neither of these genes is expressed in all tissues, their function is not required in all cells, and this is consistent with a role in a specialized process such as differentiation.

[0053] The expression pattern of both CZF-1 and -2 in the various adult tissues shows overlap with that of Sox-9 (see Fig. 9), which is a transcription factor known to regulate chondrogenesis (see Zhao, et al, 1997, Dev Dyn, 209:377-86 and U.S. Patent No. 6,143,878), suggesting a functional relationship between Sox-9 and CZF-1 and -2.

[0054] Expression of CZF-1 and -2 was examined in mouse embryonic development using Northern blotting (see Fig. 10). Maximal expression of both the 3.7 kb CZF-1, and the 4.0 kb CZF-2 transcripts was at day 11 and there was little or no expression on day 7. This pattern of expression of CZF-1 during mouse embryo development is very similar to previously published data for Sox-9, a known marker of chondrogenesis.

[0055] *In situ* hybridization of CZF-1 to a 10.5 day post-coitus whole mouse embryo (see Fig. 11) shows expression of CZF-1 in head mesenchyme, the first and second brachial arches, the forelimb bud and the tail bud, an expression pattern consistent with involvement of CZF-1 in chondrogenesis.

Isolated Polynucleotides

[0056] The present invention provides isolated polynucleotides that comprise the coding sequence of the human CZF-1 gene or a unique fragment thereof. The present invention also provides isolated polynucleotides that comprise the coding sequence of the human CZF-2 gene or a unique fragment thereof. The sequence, SEQ ID NO. 1, of a polynucleotide which comprises the full-length coding sequence of the CZF-1 gene is shown in Fig. 3. The predicted amino acid sequence, SEQ ID NO. 2, of the CZF-1 protein encoded by this polynucleotide is shown in Fig. 4. The sequence, SEQ ID NO. 3, of a polynucleotide which comprises the full-length coding sequence of the CZF-2 gene is shown in Fig. 5. The predicted amino acid sequence, SEQ ID NO. 4, of the CZF-2 protein encoded by this polynucleotide is shown in Fig. 6.

[0057] Due to the known degeneracy of the genetic code wherein more than one codon can encode the same amino acid, a DNA sequence may vary from that shown in Fig. 3 and still encode a CZF-1 protein having the amino acid sequence shown in Fig. 4. Similarly, a DNA sequence may vary from that shown in Fig. 5 and still encode a CZF-2 protein whose amino acid sequence is shown in Fig. 6.

[0058] The present invention also encompasses polynucleotides having sequences that are capable of hybridizing to the nucleotide sequences of Figs. 3 and 5 under stringent conditions, preferably highly stringent conditions. Preferably, the isolated polynucleotide

comprises a sequence which encodes a protein whose amino acid sequence is at least 90% identical, more preferably 95% identical, to the amino acid sequence of the CZF-1 protein or the CZF-2 protein shown in Figs. 4 and 6, respectively.

[0059] Hybridization conditions are based on the melting temperature TM of the nucleic acid binding complex or probe, as described in Berger and Kimmel (1987) Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press. The term "stringent conditions, as used herein, is the "stringency" which occurs within a range from about T_m-5 (5° below the melting temperature of the probe) to about 20°C below T_m. "Highly stringent hybridization conditions" refers to an overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.2x SSC at about 65°C. As recognized in the art, stringency conditions can be attained by varying a number of factors such as the length and nature, i.e., DNA or RNA, of the probe; the length and nature of the target sequence, the concentration of the salts and other components, such as formamide, dextran sulfate, and polyethylene glycol, of the hybridization solution. All of these factors may be varied to generate conditions of stringency which are equivalent to the conditions listed above.

[0060] Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lower stringency); salt conditions, or temperature. For example, moderately high stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2 M NaH_2PO_4; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 ug/ml salmon sperm blocking DNA; followed by washes at 50°C with 1X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

[0061] Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The

inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

[0062] The present invention also encompasses altered polynucleotides which encode a CZF-1 or CZF-2 protein. Such alterations include deletions, additions, or substitutions. Such alterations may produce a silent change and result in a CZF-1 or CZF-2 protein having the same amino acid sequence as the CZF-1 or CZF-2 protein encoded by the unaltered polynucleotide. Such alterations may produce a nucleotide sequence possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eucaryotic host may be incorporated into the nucleotide sequences shown in Figs. 3 and 5 to increase the rate of expression of the polypeptides encoded by such sequences. Such alterations, conventionally, are accomplished using site-directed mutagenesis.

[0063] In one embodiment of the present invention, isolated polynucleotides comprising the coding sequences of the CZF-1 or CZF-2 genes, as described above, are used as probes to hybridize with RNA extracted from cells or tissues which are suspected of containing chondrocytes. Such cells, for example, can be MPCs that have been grown in culture under conditions where differentiation into chondrocytes will occur.

[0064] Such cells can also include MPCs, or MPCs that have partially or entirely differentiated into chondrocytes, that are being prepared for implantation into human subjects that have cartilage defects for the purpose of regenerating a functional cartilage joint surface. Such cells or tissues can also be obtained from human subjects that have previously been implanted with cells that had or are suspected of undergoing chondrogenesis.

[0065] The present oligonucleotides and polynucleotides can be used to detect and characterize cells obtained from a tumor that is suspected of being a chondrosarcoma or cells that have characteristics of chondrocytes that may be present in tumor tissue. Such characterization of these cells may reveal whether the tumor cells are undifferentiated mesenchyme, or chondrocytes, for the purpose of choosing appropriate therapy in a patient from which the cells came.

[0066] The present oligonucleotides and polynucleotides could also be used to amplify or probe RNA obtained from mesenchymal cells that are being used as screening tools for compounds that are designed to accelerate or retard chondrogenesis. The present oligonucleotides and polynucleotides can be used to monitor the effect of such compounds on this process.

[0067] The present oligonucleotides and polynucleotides can also be used to probe histological sections of embryos.

[0068] The present oligonucleotides and polynucleotides can also be used to probe a biopsy sample taken from the area in the body where new bone formation is taking place after a fracture. The present oligonucleotides and polynucleotides can also be used to probe cells obtained from any other area of the body where chondrogenesis is suspected of taking place. Examples of other instances where chondrogenesis may occur include formation of osteophytes in a osteoarthritic joint and areas where repair of cartilage defects is occurring by an upwelling of cells from the subchondral bone.

[0069] In one example, the CZF-1 or CZF-2 coding sequence is radioactively labeled with ^{32}P or digoxigenin, and then hybridized in solution to RNA that is isolated from MPCs that have been grown in culture for 3 days under conditions where differentiation into chondrocytes will occur, as described above, and where the RNA has been separated by size using gel electrophoresis and blotted to nitrocellulose paper. After hybridization and washing of the nitrocellulose paper, hybridization of the CZF-1 or CZF-2 probe to RNA on the nitrocellulose, as revealed by autoradiography, indicates expression of the CZF-1 or CZF-2 genes in the cells or tissues from which the RNA was extracted.

[0070] In another embodiment of the present invention, CZF-1 or CZF-2 probes, labeled as described above, are used to hybridize directly to cells or tissues suspected of containing chondrocytes. The cells or tissues are fixed before hybridization, using procedures well known to those skilled in the art. Hybridization is performed under conditions similar to those described above. Detection of hybridization, by autoradiography for example, indicates the presence and location within the cells or tissues where CZF-1 or CZF-2 transcripts are present.

[0071] In another embodiment of the present invention, RNA is extracted from cells or tissues suspected of containing chondrocytes and is reverse transcribed into DNA. Then, polynucleotides that contain parts of the nucleotide sequence of the CZF-1 or CZF-2 genes are synthesized and used as primers in a polymerase chain reaction (PCR) to specifically amplify DNA products from the reverse transcription reaction that hybridize to the CZF-1 or CZF-2 primers. DNA products that result from such a PCR reaction indicate that the RNA, which was extracted from cells or tissue, contained mRNAs transcribed from the CZF-1 or CZF-2 genes.

Use of Oligonucleotides and Polynucleotides to Make Proteins

[0072] The polynucleotides are also useful for producing CZF-1 and CZF-2 proteins. For example, an RNA molecule encoding a CZF-1 protein is used in a cell-free translation system to prepare such protein. Alternatively, a DNA molecule encoding an CZF-2 protein is introduced into an expression vector and used to transform or transfect cells. Suitable expression vectors include, for example, chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40, bacterial plasmids, phage DNAs; yeast plasmids, vectors derived from combinations of plasmids and phage DNAs, viral DNA such as vaccinia, adenovirus, fowl pox virus, pseudorabies, baculovirus, and retrovirus. The DNA sequence is introduced into the expression vector by conventional procedures known to those skilled in the art.

[0073] Accordingly, the present invention also relates to recombinant constructs comprising one or more of the present polynucleotide sequences. Suitable constructs include, for example, vectors, such as a plasmid, phagemid, or viral vector, into which a sequence that encodes an CZF-1 protein or a CZF-2 protein has been inserted. In the expression vector, the DNA sequence which encodes the CZF-1 or CZF-2 protein is operatively linked to an expression control sequence, i.e., a promoter, which directs mRNA synthesis. Representative examples of such promoters, include the LTR or SV40 promoter, the *E. coli* lac or trp, the phage lambda PL promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or in viruses. The promoter may also be the natural promoter of the CZF-1 or CZF-2 encoding sequence. The expression vector, preferably, also contains a ribosome binding site for translation initiation and a transcription terminator. Preferably, the recombinant expression vectors also include an origin of replication and a selectable marker, such as for example, the

ampicillin resistance gene of *E. coli* to permit selection of transformed cells, i.e. cells that are expressing the heterologous DNA sequences. The polynucleotide sequence encoding the CZF-1 or CZF-2 protein is incorporated into the vector in frame with translation initiation and termination sequences.

[0074] The polynucleotides encoding an CZF-1 or CZF-2 protein are used to express recombinant protein using techniques well known in the art. Such techniques are described in Sambrook, J. et al (1989) Molecular Cloning A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y. and Ausubel, F. M. et al. (1989) Cuurent Protocols in Molecular Biology, John Wile & Sons, New York, NY.

[0075] Also encompassed by the present invention, are single stranded polynucleotides, hereinafter referred to as antisense polynucleotides, having sequences which are complementary to the DNA and RNA sequences which encode the CZF-1 and CZF-2 proteins. The term complementary as used herein refers to the natural binding of the polynucleotides under permissive salt and temperature conditions by base pairing.

[0076] The present invention also encompasses oligonucleotides that are used as primers in polymerase chain reaction (PCR) technologies to amplify transcripts of the genes which encode CZF-1 and CZF-2 proteins or portions of such transcripts. Preferably, the primers comprise 18-30 nucleotides, more preferably 19-25 nucleotides. Preferably, the primers have a G+C content of 40% or greater. Such oligonucleotides are at least 98% complementary with a portion of the DNA strand, i.e., the sense strand, which encodes the respective CZF protein or a portion of its corresponding antisense strand. Preferably, the primer has at least 99% complementarity, more preferably 100% complementarity, with such sense strand or its corresponding antisense strand. Primers which are which have 100% complementarity with the antisense strand of a double-stranded DNA molecule which encodes an CZF protein have a sequence which is identical to a sequence contained within the sense strand. The identity of primers which are 15 nucleotides in length and have full complementarity with a portion of the antisense strand of a double-stranded DNA molecule which encodes the CZF-1 and -2 proteins is determined using the nucleotide sequences, shown in Figs. 3 and 5.

[0077] The present invention also encompasses oligonucleotides that are useful as hybridization probes for isolating and identifying cDNA clones and genomic clones encoding the CZF-1 or CZF-2 protein or allelic forms thereof. Such hybridization probes are also useful for detecting transcripts of the genes which encode the CZF-1 or CZF-2 protein or for mapping of the genes which encode the CZF-1 or CZF-2 proteins. Preferably, such oligonucleotides comprise at least 210 nucleotides, up to 500 nucleotides in length, more preferably at least 230, most preferably from about 210 to 280 nucleotides. Such hybridization probes have a sequence which is at least 90% complementary with a sequence contained within the sense strand of a DNA molecule which encodes an CZF-1 or CZF-2 protein or with a sequence contained within its corresponding antisense strand. Such hybridization probes bind to the sense strand under stringent conditions. The probes are used in Northern assays to detect transcripts of CZF-1 or CZF-2 homologous genes, and in Southern assays to detect CZF-1 or CZF-2 homologous genes. The identity of probes which are 200 nucleotides in length and have full complementarity with a portion of the antisense strand of a double-stranded DNA molecule which encodes the CZF-1 or CZF-2 protein is determined using the nucleotide sequences shown in Figs. 3 and 5, respectively, and described by the general formula a-b; where a is any integer between 1 and the position number of the nucleotide which is located 200 residues upstream of the 3' end of the sense or antisense strand of the cDNA sequences shown in Figs. 3 and 5; b is equal to a +200; and where both a and b correspond to the positions of nucleotide residues of the cDNA sequences shown in Figs. 3 and 5. In a preferred embodiment, the oligonucleotide for CZF-1 comprises a sequence which encompasses nucleotide 476 through nucleotide 939 of the sequence shown in Fig. 3. This is the spacer regionbetween the KRAB B domain and the ZF repeats (see Fig. 4). In a preferred embodiment, the oligonucleotide for CZF-2 comprises a sequence which encompasses nucleotide 163 through nucleotide 423 of the sequence shown in Fig. 5. This is the spacer region between the KRAB A domain and the ZF repeats (see Fig. 6).

[0078] Such probes or primers are also useful for identifying tissues or cells in which the corresponding CZF gene is expressed. Expression of the CZF-1 or CZF-2 gene in a particular tissue or group of cells is determined using conventional procedures including, but not limited to, Northern analysis, in situ hybridization to RNA or RT-PCR amplification. Isolated polynucleotides encoding a CZF-1 or CZF-2 protein are also useful as chromosome markers to map linked gene positions, to identify chromosomal aberrations such as translocations,

inversions and trisomies, to compare with endogenous DNA sequences in patients to identify potential genetic disorders, and as probes to hybridize and thus discover novel, related DNA sequences. For use in such studies and assays, the probes may be labeled with radioisotopes, fluorescent labels, or enzymatic labels. The assays include, but are not limited to, Southern blot, in situ hybridization to DNA in cells and chromosomes, PCR, and allele specific hybridization.

Antibodies

[0079] In another aspect, the present invention relates to antibodies which are specific for and bind to the CZF-1 or CZF-2 protein. Such antibodies are useful research tools for identifying tissues that contain normal or elevated levels of the respective protein and for purifying the respective protein from cell or tissue extracts, medium of cultured cells, or partially purified preparations of intracellular and extracellular proteins by affinity chromatography. Such antibodies are also useful for identifying and diagnosing diseases associated with elevated or reduced levels of the CZF-1 or CZF-2 protein. Such antibodies are also useful for monitoring the effect of therapeutic agents on the synthesis of the CZF proteins by cells *in vitro* and *in vivo*. Such antibodies may also be employed in procedures, such as co-immunoprecipitation and co-affinity chromatography, for identifying other proteins, activators and inhibitors which bind to the CZF-1 or CZF-2 protein.

[0080] The present invention also provides a method for detecting a CZF-1 or CZF-2 protein, in a bodily sample from a patient using antibodies immunospecific for the CZF-1 protein or CZF-2 protein, respectively. The method comprises contacting the antibody with a sample taken from the patient; and assaying for the formation of a complex between the antibody and the corresponding CZF protein present in the sample. The sample may be a tissue or a biological fluid, including but not limited to whole blood, serum, synovial fluid, stool, urine, cerebrospinal fluid, semen, tissue biopsies or excised tissue or cells obtained from swabs and smears. To monitor changes in expression of the CZF protein during fetal development and pregnancy, it is preferred that the sample be amniotic fluid. To monitor changes in expression of the CZF protein during joint disorders, the preferred sample is synovial fluid. To monitor changes in expression of the CZF protein during cancer, the preferred samples is biopsy tissue or blood..

[0081] The sample may be untreated, or subjected to precipitation, fractionation, separation, or purification before combining with the anti-CZF protein antibody. For ease of detection, it is preferred that isolated proteins from the sample be attached to a substrate such as a column, plastic dish, matrix, or membrane, preferably nitrocellulose. Preferably, the detection method employs an enzyme-linked immunosorbent assay (ELISA) or a Western immunoblot procedure.

[0082] Interactions between an CZF protein in the sample and the corresponding anti-CZF antibody are detected by radiometric, colorimetric, or fluorometric means, size separation, or precipitation. Preferably, detection of the antibody-CZF protein complex is by addition of a secondary antibody that is coupled to a detectable tag, such as for example, an enzyme, fluorophore, or chromophore. Formation of the complex is indicative of the presence of the CZF protein in the test sample. Thus, the method is used to determine whether there is a decrease or increase in the levels of the CZF protein in a test sample as compared to levels of the CZF protein in a control sample and to quantify the amount of the CZF protein in the test sample. Deviation between control and test values establishes the parameters for diagnosing the disease.

Preparing the CZF protein

[0083] The present invention relates to novel, isolated, substantially purified, mammalian proteins. As used herein, the term "substantially purified" refers to a protein that is removed from its natural environment, isolated or separated, and at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

[0084] The novel mammalian proteins are CZF-1 and CZF-2. In one embodiment, the CZF-1 protein is a human protein which comprises amino acids 306-717 of the amino acid sequence set forth in SEQ ID NO. 2. In one embodiment, the CZF-2 protein is a human protein which comprises the amino acid sequence set forth in SEQ ID NO. 4.

[0085] CZF-1 is a Cys_2His_2-class zinc-finger protein having at its N-terminus, KRAB-A and KRAB-B domains, a spacer region and 16 zinc-finger domains. The molecular mass is 82691.75. CZF-2 is also a Cys_2His_2-class zinc-finger protein having at its N-terminus a KRAB A domain, a spacer region, and 12 zinc-finger domains. It has a molecular mass of 59753.89.

The KRAB motif is found in numerous zinc finger proteins of this class, and usually indicates that the molecule functions as a transcriptional repressor. The zinc-finger domains act as DNA binding domains, and interact with specific sequences in genomic DNA. As determined using the BLAST software from the National Center for Biotechnology Information, the predicted CZF-1 and CZF-2 proteins show an overall 20-30% similarity to each other, although this may be considerably higher or lower for individual domains as described below.

[0086] The CZF-1 and CZF-2 also encompass variants of the CZF proteins shown in Figs. 4 and 6 respectively. A "variant" as used herein, refers to a protein whose amino acid sequence is similar to one of the amino acid sequences shown in Figs. 4 and 6, hereinafter referred to as the reference amino acid sequence, but does not have 100% identity with the reference sequence. The variant protein has an altered sequence in which one or more of the amino acids in the reference sequence is deleted or substituted, or one or more amino acids are inserted into the sequence of the reference amino acid sequence. As a result of the alterations, the variant protein has an amino acid sequence which is at least 95% identical to the reference sequence, preferably, at least 97% identical, more preferably at least 98% identical, most preferably at least 99% identical to the reference sequence. Variant sequences which are at least 95% identical have no more than 5 alterations, i.e. any combination of deletions, insertions or substitutions, per 100 amino acids of the reference sequence. Percent identity is determined by comparing the amino acid sequence of the variant with the reference sequence using MEGALIGN project in the DNA STAR program. Sequences are aligned for identity calculations using the method of the software basic local alignment search tool in the BLAST network service (the National Center for Biotechnology Information, Bethesda, MD) which employs the method of Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403-410. Identities are calculated by the Align program (DNAstar, Inc.) In all cases, internal gaps and amino acid insertions in the candidate sequence as aligned are not ignored when making the identity calculation.

[0087] While it is possible to have nonconservative amino acid substitutions, it is preferred that the substitutions be conservative amino acid substitutions, in which the substituted amino acid has similar structural or chemical properties with the corresponding amino acid in the reference sequence. By way of example, conservative amino acid substitutions involve

substitution of one aliphatic or hydrophobic amino acids, e.g. alanine, valine, leucine and isoleucine, with another; substitution of one hydroxyl-containing amino acid, e.g. serine and threonine, with another; substitution of one acidic residue, e.g. glutamic acid or aspartic acid, with another; replacement of one amide-containing residue, e.g. asparagine and glutamine, with another; replacement of one aromatic, residue, e.g. phenylalanine and tyrosine, with another; replacement of one basic residue, e.g. lysine, arginine and histidine, with another; and replacement of one small amino acid, e.g., alanine, serine, threonine, methionine, and glycine, with another.

[0088] The alterations are designed not to abolish the immunoreactivity of the variant protein with antibodies that bind to the reference protein. Guidance in determining which amino acid residues may be substituted, inserted or deleted without abolishing immunoreactivity of the variant protein with an antibody specific for the respective reference protein are found using computer programs well known in the art, for example, DNASTAR software.

[0089] The CZF proteins also encompass fusion proteins comprising an CZF-1 or CZF-2 protein and a tag, i.e., a second protein or one or more amino acids, preferably from about 2 to 65 amino acids, more preferably from about 34 to about 62 amino acids, which are added to the amino terminus of, the carboxy terminus of, or any point within the amino acid sequence of the respective CZF proteins, or a variant of such protein. Typically, such additions are made to stabilize the resulting fusion protein or to simplify purification of an expressed recombinant form of the corresponding CZF protein. Such tags are known in the art. Representative examples of such tags include sequences which encode a series of histidine residues, the epitope tag FLAG, the Herpes simplex glycoprotein D, beta-galactosidase, maltose binding protein, or glutathione S-transferase.

[0090] The CZF proteins also encompass CZF-1 or CZF-2 proteins in which one or more amino acids, preferably no more than 10 amino acids, in the respective CZF protein are altered by posttranslation processes or synthetic methods. Examples of such modifications include, but are not limited to, acetylation, amidation, ADP-ribosylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or a lipid, cross-linking gamma-carboxylation, glycosylation, hydroxylation, iodination, methylation,

myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, sulfation, and transfer-RNA mediated additions of amino acids to proteins such as arginylation and ubiquitination.

[0091] The CZF protein, may be produced by conventional peptide synthesizers. The CZF protein may also be produced using cell-free translation systems and RNA molecules derived from DNA constructs that the CZF protein. Alternatively, the CZF protein is made by transfecting host cells with expression vectors that comprise a DNA sequence that encodes the respective CZF protein and then inducing expression of the protein in the host cells. For recombinant production, recombinant constructs comprising one or more of the sequences which encode the CZF proteins are introduced into host cells by conventional methods such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape lading, ballistic introduction or infection.

[0092] The CZF-1 and CZF-2 proteins may be expressed in suitable host cells, such as for example, mammalian cells, yeast, bacteria, insect cells or other cells under the control of appropriate promoters using conventional techniques. Suitable hosts include, but are not limited to, E. coli, P. pastoris, Cos cells and 293 HEK cells. Following transformation of the suitable host strain and growth of the host strain to an appropriate cell density, the cells are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification of the CZF protein.

[0093] Conventional procedures for isolating recombinant proteins from transformed host cells, such as isolation by initial extraction from cell pellets or from cell culture medium, followed by salting-out, and one or more chromatography steps, including aqueous ion exchange chromatography, size exclusion chromatography steps, and high performance liquid chromatography (HPLC), and affinity chromatography may be used to isolate the recombinant CZF-1 protein or CZF-2 protein.

Preparation of Antibodies

[0094] The CZF proteins thereof are used as immunogens to produce antibodies immunospecific for one or more CZF protein. The term "immunospecific" means the antibodies have substantially greater affinity for the CZF protein than for other proteins. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, and Fab fragments.

[0095] Antibodies are also prepared using an oligopeptide having a sequence which is identical to a portion of the amino acid sequence of an CZF protein. Preferably the oligopeptide has an amino acid sequence of at least five amino acids, and more preferably, at least 10 amino acids that are identical to a portion of the amino acid sequence of an CZF protein. Such peptides are conventionally fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule. Such peptides can be determined using software programs, for example the MacVector program, to determine hydrophilicity and hydrophobicity and ascertain regions of the protein that are likely to be present at the surface of the molecule.

[0096] Polyclonal antibodies are generated using conventional techniques by administering the CZF protein or a chimeric molecule to a host animal. Depending on the host species, various adjuvants may be used to increase immunological response. Among adjuvants used in humans, Bacilli-Calmette-Guerin (BCG), and *Corynebacterium parvum.* are especially preferable. Conventional protocols are also used to collect blood from the immunized animals and to isolate the serum and or the IgG fraction from the blood.

[0097] For preparation of monoclonal antibodies, conventional hybridoma techniques are used. Such antibodies are produced by continuous cell lines in culture. Suitable techniques for preparing monoclonal antibodies include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV hybridoma technique.

[0098] Various immunoassays may be used for screening to identify antibodies having the desired specificity. These include protocols which. involve competitive binding or

immunoradiometric assays and typically involve the measurement of complex formation between the respective CZF protein and the antibody.

Polynucleotides that Encode the CZF-1 and CZF-2 Proteins

[0099] Polynucleotides comprising sequences encoding a CZF-1 or CZF-2 protein may be synthesized in whole or in part using chemical methods. Polynucleotides which encode a CZF-1 or CZF-2 protein, particularly alleles of the genes which encode these two proteins, may be obtained by screening a genomic library or cDNA library with a probe comprising sequences identical or complementary to the sequences shown in Figs. 3 and 5, or with antibodies immunospecific for a CZF-1 or CZF-2 protein to identify clones containing such polynucleotide.

EXAMPLES

[0100] The following examples are for purposes of illustration only and are not intended to limit the scope of the invention as defined in the claims which are appended hereto. The references cited in this document are specifically incorporated herein by reference.

Example 1. - Growth and Differentiation of Mesenchymal Cells

[0101] MSCs were obtained from bone marrow obtained from the iliac crests (hip) of patients undergoing spine surgery. The cells were plated at 10 x 10^6 nucleated cells/100 mm cell culture plate after fractionation on a Percoll gradient. Cells from the gradient that adhered to the culture plate formed colonies that covered about 80-90% of the plate by 14-20 days in monolayer culture. The cells were then trypsinized, counted and 2 x 10^5 cell aliquots were centrifuged at 500 x g for 5 minutes in 15 ml polypropylene tubes in 0.5 ml of a defined medium consisting of DMEM-high glucose with ITS + Premix: insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 μg/ml) and linoleic acid (5.35 μg/ml) with bovine serum albumin (1.25 μg/ml), and pyruvate (1 mM), ascorbate 2-phosphate (37.5 μg/ml), dexamethasone (10^{-7} M) and TGFβ-1 (10 ng/ml, recombinant human). The pelleted cells were incubated at 37°C, 5% CO_2. Within 24 hours of incubation, the cells formed an aggregate that did not adhere to the walls of the tube. Typically, medium changes were carried out at 2-3 day intervals and aggregates were be

harvested at time points up to 21 days. However, cell aggregates were isolated as early as 1 day of culture.

Example 2. - Isolation of RNA from Cell Aggregates, Preparation of cDNA Library and Library Screening

[0102] RNA was prepared from the cells of Example 1 that had been in culture for 3 days, and used to construct a cDNA library in the λgt10 vector using standard methods well known to those in the art. This library was screened, using a ^{32}P-labeled degenerate oligonucleotide probe, coding for the HTGEKP sequence (5'-CA(CT) AC(ACTG) GG(ACTG) GA(AG) AA(AG) CC(ATCG)-3', SEQ ID NO. 5). Cloned cDNA inserts from λgt10 clones that hybridized to the oligonucleotide probe were amplified from hybridizing plaques by PCR using LD insert screening amplimers (Clontech) as primers. Inserts were cloned directly into the pCR®2.1 plasmid vector (Invitrogen).

[0103] These cloned inserts were then sequenced. Sequence were assembled and analyzed using AssemblyLign and MacVector software (Oxford Molecular Group). CZF-1 was a cDNA of 1485 bp, containing a 412 amino acid residue open reading frame coding for a protein containing 14 zinc-finger domains CZF-2 was a 2166 bp sequence containing a 518 amino acid open reading frame, containing 12 zinc-finger domains.

[0104] All of these procedures are well known and routine to one skilled in the art.

Example 3. - Characterization of CZF-1 and CZF-2 Clones

[0105] The DNA sequences were analyzed using the BLASTX program at NCBI (http://www.ncbi.nlm.nih.gov/). All databases including dbEST, dbSTS, and the non-redundant database were searched.

[0106] A BLAST search revealed identity of CZF-1 with sequences within a 3.6 Mb region in 19q13.4, as well as an additional EST isolated from an ovary cDNA library. A BLAST search revealed that the sequence of CZF-2 matched ESTs derived from human bone marrow stromal cells, infant brain and neuro-epithelium cDNA libraries.

Example 4. - Expression of CZF-1 and Collagen Types II and X in MPCs Undergoing Chondrogenesis

[0107] Total cellular RNA was isolated from MPCs that had been grown in culture for different periods (day 0 through day 21 of aggregate culture) under conditions where differentiation to chondrocytes occurred (see Example 1).). Quantitiative PCR was carried out using the Lightcycler instrument and the LightCycler-FastStart DNA Master SYBR Green I kit from Roche.

Example 5. - Expression of CZF-1 and -2, and Sox-9 in Multiple Mouse Tissues

[0108] Multiple human tissue and mouse embryo developmental stage-specific Northern membranes, purchased from Clontech, were probed with random primer-radiolabelled restriction fragments of the full-length inserts using standard techniques.

Example 6. -Characterizing Chondrogenesis in Chick Embryos Using Polynucleotides Derived from CZF-1 or CZF-2 or Anti- CZF-1 or Anti-CZF-2 Antibodies.

WHOLE-MOUNT IN SITU HYBRIDISATION ON CHICKEN EMBRYOS

[0109] (Chitnis et al., 1995; Henrique et al., 1995)

[0110] Domingos Henrique and David Ish-Horowicz. (ICRF Dev. Biol. Unit, Oxford; Fax 0865 281310)

[0111] Modified from protocols of Ron Conlon (Mt. Sinai, Toronto), Phil Ingham (ICRF Oxford) and David Wilkinson (NIMR, London). Note hybridisation in much reduced salt concentration and omission of RNAse digestion. New conditions for antibody detection are derived from Harland's lab protocol for frogs. Also tried on mouse embryos (7.5-10.5 dpc), and Xenopus embryos, successfully.

DISSECTIONS

[0112] 1. Dissect embryos out in PBS + 2mM EGTA; remove as much of the extra-embryonic membranes as possible.

[0113] 2. Fix in 10ml 4% formaldehyde (HCHO) in PBS + 2mM EGTA, 1-2h at room temp; or 4°C, 2h.

[0114] 3. Wash three times in PTW (= PBS, 0.1% Tween-20) and once for 1 hour.

[0115] 4. Transfer to 100% MeOH; can store at this point at -20°C (less than one month).

PRETREATMENTS and HYBRIDIZATION

[0116] 5. Rehydrate embryos through 75%, 50%, 25% MeOH/PTW (allowing embryos to settle), and washing three times with PTW.

[0117] 6. Treat with 10μg/ml proteinase K in PTW for 7 minutes at 37°C (prewarmed solutions!).

[0118] 7. Remove proteinase, rinse twice briefly (carefully and quickly!) with PTW, and post-fix for 20 min in 4% HCHO + 0.1% Glutaraldehyde, in PTW.

[0119] 8. Rinse and wash once with PTW.

[0120] 9. Rinse once with 1:1 PTW/hybridisation mix. Let embryos settle.

[0121] 10. Rinse with 1 ml hybridisation mix. Let embryos settle.

[0122] 11. Replace with 1 ml hybridisation mix and incubate with gentle mixing ≥1h/65°C.

[0123] [Can store at -20°C (before or) after prehybridising.]

[0124] 12. Add 1ml pre-warmed hybridisation mix @ ~1μg/ml DIG-labelled RNA probe (possibly 0.1 μg/ml is enough)

[0125] 13. Incubate with gentle mixing at 65°C/overnight.

[0126] •Steps 1-4 are carried out in 15 ml falcon tubes, subsequent steps in 1.7-2ml in a 2ml microtube rocking at room temperature unless otherwise stated. Unless otherwise stated, rinses are immediate, and washes are for 5 min.

[0127] •A stock of 8% glutaradehyde is stored in aliquots at -20°C. Thaw out aliquot just before use.

Hybridization mix:

Formamide	50%	25ml	
SSC (20x pH 5 w citric acid!!)	**1.3xSSC**	3.25ml	
EDTA (0.5M, pH8)	5mM	0.5ml	
Yeast RNA (20mg/ml)	50µg/ml	125µl	
Tween-20 (10%)	0.2%	1ml	
CHAPS (10%)	0.5%	2.5ml	
Heparin (50mg/ml)	100µg/ml	100µl	
H_2O		17.5ml	
Total		50ml	

POST-HYBRIDIZATION WASHES

[0128] 1. Rinse twice with prewarmed (65°C) hybridization mix.

[0129] 2. Wash 10 min/65°C with prewarmed hyb mix.

[0130] 3. Wash 2x30 min/65°C with Washing solution 1 (50% Formamide/1xSSC/ 0.1%Tween-20), prewarmed at 65°C.

[0131] 4. Wash 10 min/65°C with prewarmed 1:1 Washing solution 1/Maleic Acid Buffer (**MABT**: 100mM maleic acid [Sigma M0375], 150 mM NaCl, 0.1% Tween-20, final pH 7.5).

[0132] 5. Rinse 3 times with MABT.

[0133] 6. Wash 2 x 30min with MABT.

[0134] 7. Replace with MABT + 2% **BBR** (**B**oehringer **B**locking **R**eagent [BM 1096 176], make 10% stock in MAB by heating to dissolve, autoclave, aliquot and freeze).

[0135] Wash for 1 hour at room temp.

[0136] 8. Preincubate in 2 ml of MABT+ 2% BBR + 20% heat treated goat serum (65^{0}C for 30 min), for 1-2 hours.

[0137] 9. Replace with a solution of MABT + 2% BBR + 20% serum, containing a 1/5000 dilution of anti-DIG-AP antibody (BM 1093 274). Incubate overnight at +4^{0}C.

[0138] • After each 70°C wash, let embryos settle by incubating tube vertically at 70°C, then change supernatants individually so samples don't cool. Keep wash solutions at 70°C in water-bath.

[0139] • Serum is heat-treated at 65°C, 30 min and stored in quick-frozen aliquots at –20°C. Thawed aliquots can be stored at 4°C with addition of azide to 0.1%.

[0140] • If using a probe labelled with UTP-fluorescein, instead of UTP-DIG, use a 1/8000 dilution of the anti-fluorescein-AP antibody (BM 1426 338).

POST-ANTIBODY WASHES AND HISTOCHEMISTRY

[0141] 1. Rinse 3 times with MABT. Transfer to scintillation vial.

[0142] 2. Wash 3x1h with 10-20 ml MABT, by rolling. If desired, washing without rocking can proceed overnight for lower background.

[0143] 3. Wash 3x10 min with NTMT.

[0144] 4. Incubate with 1.5ml NTMT + 4.5µl/ml NBT + 3.5µl/ml BCIP. Rock for first 20min. (Develops faster at 37°C, if necessary)

[0145] 5. When colour has developed to the desired extent (30min to 3 days), wash 3x with PTW. Refix in 4% HCHO/0.1% Glutaraldehyde/PTW, overnight, followed by PTW washes and storage in PTW/0.1% azide, at +4°C.

[0146] 6. Clear in 50% glycerol/PTW then 80% glycerol/PTW/0.02% azide.

NTMT:		
	5M NaCl	1ml
	2M TrisHCl pH9.5	2.5ml
	2M $MgCl_2$	1.25ml
	10% Tween-20	5ml
	H_20	40.25ml
	Total	50ml

Make from stocks on day of use.

N.B. Tween-20 final concentration is 1%.

REFERENCES

[0147] Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature 375, 761-766.

[0148] Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. Nature 375, 787-790.

[0149] Make 20% paraformaldehyde fresh before use: Heat 17 ml of water on a hot plate with stirring. Add 1 drop of 10 N NaOH, then 4 g of paraformaldehyde and stir until dissolved. Make up to 20 ml.

[0150] 10X PBS (100 ml) 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4

Example 7 - Hybridization to Sections of Mouse Embryos

[0151] Whole mouse sagitally sectioned sections mounted on slides were obtained from day 10.5 post-coitus embryos (obtained from Parogon Biothech, Inc., Baltimore, MD) and were probed with CZF-1 or Sox-9 cDNAs which had been cloned into a plasmid allowing *in vitro* transcription. The plasmids were linearized to produce an appropriate blunt or 3'recessed end, and transcribed with T7, T3 or SP6 RNA polymerase to generate digoxigenin-labelled sense and antisense probes using a commercially available kit (Genius 4 system (Boehringer Mannheim, Indianapolis, IN).

[0152] After digestion with proteinase K, the sections were washed in PBS and fixed in 4% paraformaldehyde-PBS. Digoxigenin-labelled probes were dissolved in hybridization buffer (50% formamide, 10 mM Tris-HCl, pH 7.6, 200 mg/ml yeast tRNA, 1X Denhardt's, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, pH 8.0). The hybridization mix was be boiled and applied directly onto sections. After hybridization for 12-16 hrs. at 53°C, the sections were washed in 5X SSC at 56°C for 1 min, 2X SSC at 56°C for 30 min, TNE (10 mM TRIS, pH 7.5, 0.5 M NaCl, 1 mM EDTA) at 37°C for 10 min. Sections were digested with 10 μg/ml RNAse A in TNE at 37°C for 30 min., followed by washing with TNE at 37°C for 10 min, 2X SSC at 53°C for 20 min, and 0.2X SSC at 53°C, twice for 20 min. Sections were blocked using 2% BBR (Boehringer Blocking Reagent) in DIG1 buffer (100 mM Tris-HCl, pH 7.5, 150

mM NaCl). Following a rinse in DIG1 buffer, sections were incubated in a dilution of anti-DIG antibody in DIG1 buffer for 1 hr., washed in DIG1 buffer, and reacted with NTB and X-phos in DIG3 buffer (100 mM Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$)overnight in the dark. Slides were rinsed in TE buffer and distilled water, counterstained with methyl green and mounted with Permount.

Example 8. - Hybridization to Tissue Samples

[0153] A tissue sample taken from a human subject that had been previously implanted with chondrocytes or MPCs. Then, biopsy to get cells/tissue from the area. Then hybridization as in Example 7.